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Tumorigenesis and Neoplastic Progression

Tumor Cell Cross Talk with Tumor-Associated Leukocytes Leads to Induction of Tumor Exosomal Fibronectin and Promotes Tumor Progression

ZhongBin Deng,* Ziqiang Cheng,†‡ Xiaoyu Xiang,* Jun Yan,§ Xiaoying Zhuang,* Cunren Liu,† Hong Jiang,* Songwen Ju,* Lifeng Zhang,* William Grizzle,† James Mobley,¶ Jesse Roman,§ Donald Miller,* and Huang-Ge Zhang*‖

From the Department of Research & Development, Robley Rex Medical Center, Louisville, Kentucky; the Department of Microbiology & Immunology,† the Brown Cancer Center, and the Department of Medicine,§ University of Louisville, Louisville, Kentucky; the Departments of Pathology‡ and Pharmacology and Toxicology,‖ University of Alabama at Birmingham, Birmingham, Alabama; and the Department of Microbiology,¶ College of Animal Science and Veterinary Medicine, Shandong Agricultural University, Tai'an, China*

Exosomes participate in intercellular communication, but most data published are based on exosomes released from *in vitro* cultured cells that do not communicate with neighboring cells located in the same microenvironment as the exosomal-producing cells *in vivo*. In this study, our data show that co-culture of leukocytes isolated from breast tumor tissue leads to uptake of fibronectin (FN) on or in the tumor exosomes (Exo^{fib+}). The induction of FN and exosomal uptake is tumor tissue derived and leukocyte specific, because leukocytes isolated from the peripheral blood of naïve mice failed to induce FN uptake by tumor exosomes. Furthermore, depletion of both $CD25^+$ cells and Gr-1^+ cells from tumor-associated leukocytes causes a reduction of Exo^{fib+}, suggesting that tumor-associated $CD25^+$ cells and Gr-1^+ cells participate in FN production and uptake by tumor exosomes, resulting in Exo^{fib+}. As a result of tumor cells absorbing Exo^{fib+}, two major events are induced: focal adhesion kinase/Src-dependent signaling pathways are activated, and the production of proinflammatory cytokines and metalloproteinase 9 is enhanced in response to absorbing exosomes. This, in turn, enhances tumor cell invasion *in vitro* and *in vivo*. Collectively, our findings provide evidence that exosomes released from freshly excised tumor tissue cells that have communicated/interacted with immune cells gain new immune evasion capacity. ***(Am J Pathol 2012, 180:390–398; DOI: 10.1016/j.ajpath.2011.09.023)***

Exosomes are small cellular vesicles that are secreted from many cell types that transfer proteins, mRNAs, and microRNAs to neighboring or distant cells to modulate immune function, angiogenesis, cell proliferation, tumor cell invasion, and cell-to-cell communication.[1] When produced by malignant cells, exosomes can promote angiogenesis, cell proliferation, tumor cell invasion, and immune evasion.[2–4] However, most of the results from recent research were obtained using tumor exosomes isolated from the supernatants of *in vitro* cultured tumor cells. Whether exosomes released from tumor cells that have interacted with leukocytes infiltrating a tumor play a role in tumor progression and metastasis has not been addressed, and whether involvement of immune cells in the regulation of tumor exosome–mediated tumor progression occurs remains uncertain.

Recent work has revealed that, in addition to tumor cells themselves, the tumor microenvironment is composed of a variety of host-derived cells, such as $CD25^+$ regulatory T cells (Tregs)[5–11] and Gr-1^+ myeloid-derived suppressor cells,[12–15] endothelial cells, and fibroblasts. The tumor microenvironment is a complex, highly dynamic environment that participates in tumor development and progression. Clinical and experimental studies have established long-term infiltration of neoplastic tissue by leukocytes that cause chronic inflammation and pro-

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Z.D. and Z.C. contributed equally to this work.

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Address reprint requests to Huang-Ge Zhang, M.D., Ph.D., Brown Cancer Center, University of Louisville, Clinical Transitional Research Building 309, 505 Hancock St, Louisville, KY 40202. E-mail: h0zhan17@louisvile.edu.

mote development and/or progression of solid tumors. However, the cellular and molecular pathways of leukocytes that are pro-tumor progression, as opposed to anti-tumor immunity, are incompletely understood.

Fibronectin (FN) controls the growth and survival of tumor cells. FN signaling is transmitted via integrins that eventually determine the cellular response to the changes in cell shape, mobility, and proliferation. Several FN receptors (integrins) bind FN to anchor cells, subsequently activating the nonreceptor tyrosine kinases, focal adhesion kinase (FAK), and Src, which all play an important role in tumorigenesis by promoting the proliferation and invasion of cancer cells.[16–22]

In this study, we established that FN is recruited into or absorbed by tumor exosomes after co-culture of tumor cells with leukocytes that infiltrated the tumor. The neutralization of exosomal FN or small-interfering RNA (siRNA) knockdown of FN in tumor exosome–producing cells also partially reverses the tumor exosome–mediated tumor cell invasion. Furthermore, we found that, unlike tumor exosomes released from *in vitro* cultured tumor cells, co-culture of tumor cells with leukocytes that had infiltrated tumor led to more aggressive tumor invasion and tumor metastasis. The depletion of $CD25^+$ cells and $Gr\text{-}1^+$ cells from tumor-infiltrating leukocytes resulted in the attenuation of tumor exosome–mediated tumor invasion and tumor metastasis. These findings suggest that the effects of tumor-associated leukocytes on tumor cells also modulate tumor exosomal content and properties. The exosomes may manipulate the tumor microenvironment to influence the invasiveness of tumor cells and, perhaps, may have adverse effects on neighboring cells through the FN-integrin signaling pathway.

Materials and Methods

Tumor Cell Invasion Assay

For analysis of cell invasion, tumor cells were preincubated with tumor cell exosomes [10 μg/1 $\times$ 10^6 tumor cells in 1 mL of Dulbecco's modified Eagle's medium (DMEM)] for 30 minutes at 37°C. Cells were then washed extensively with DMEM three times. Washed cells were finally suspended in DMEM media with 10% fetal bovine serum (1 $\times$ 10^6 cells/mL). Analysis of cell migration was performed using 8.0-μm transwell chambers (8-μm pore size; Costar, Cambridge, MA). The upper transwell chamber was coated with 5 mg of Matrigel (Collaborative Research, Bedford, MA), diluted in cold water, and dried at 22°C. Before use, the transwells were incubated with DMEM, and the tumor cells (1 $\times$ 10^5/well) pretreated with exosomes were added to the upper chamber for 24 hours at 37°C. Cells that had invaded or migrated to the lower surface of the membrane were fixed with methanol, stained with DAPI, viewed using fluorescence microscopy, and quantified by counting the number of stained nuclei in five individual fields in each transwell filter.

In Vivo *Metastasis Model*

To prepare 4T1 exosomes to be used for treatment of 4T1-Luc cells, 4T1 cells were first incubated at 37°C for 1 hour with 4T1 exosomes (10 μg/1 $\times$ 10^6 4T1-Luc) in the following combinations: exosomes isolated from 4T1 cells after being co-cultured with tumor-associated leukocytes with or without the depletion of CD25, Gr1, or CD25 plus Gr1 cells; and after being co-cultured with tumor-associated leukocytes, 4T1 exosomes isolated from 4T1 cells were preincubated with anti-FN extra domain A (EDA) (1 μg/mg of 4T1 exosomes) at 22°C for 30 minutes. After a 1-hour incubation, 4T1 cells were washed with three times DMEM. The washed cells were then suspended in DMEM media for injecting i.v. into BALB/c mice. All experiments involving animals were approved by the University of Louisville Institutional Animal Care and Use Committee (Louisville, KY). Female BALB/c mice (aged 6 to 8 weeks) were tail-vein injected with the 4T1-Luc (5 $\times$ 10^5/mouse) stable cell line, as previously prepared and described. On days 5 and 10 after i.v. injection, animals were analyzed for metastatic disease by bioluminescence imaging using an IVIS-100 camera system for the detection of luciferase expression (Xenogen, Alameda, CA). Briefly, mice were anesthetized with isoflurane and injected i.p. with 2.2 mg of luciferin sodium salt (GOLD Bio Technology, Inc., St. Louis, MO) in PBS (pH 7.4). Each image was sequentially acquired three to four times, and data were collected at the time of peak luminescence. The bioluminescence images were overlaid on black-and-white photographs of the mice, collected at the same time. The signal intensity was quantified as the sum of all detected photon counts within a region of interest using Living Image software, version 2.50 (Xenogen).

Cell Culture

Mouse 4T1 and mouse CT26 colon cancer cell lines were obtained from American Type Culture Collection (Manassas, VA). The AT-3 breast tumor cell line was provided by Dr. Jeffrey Schlom (Laboratory of Tumor Immunology and Biology, National Cancer Institute, Bethesda, MD). All cell lines were maintained in DMEM (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum (Invitrogen), penicillin, and streptomycin (Invitrogen). Transient transfections of siRNA were performed using Fugene HD (Roche, Indianapolis, IN).

Production of Exosomes from Co-Cultured Tumor Cells with Tumor-Associated Leukocytes

Mice received 1.2 $\times$ 10^5 tumor cells in 50 μL of PBS in the flank regions. Tumor tissue was removed from mice on day 15 after injection. Tumor tissue was digested with 1 mg/mL collagenase I (Worthington, Lakewood, NJ) and 0.05 mg/mL DNase (Sigma-Aldrich, St. Louis, MO) using a previously described method.[23] After dissociation of the tumor cells, the tumor-associated leukocytes were purified from digested tumor tissue by centrifugation in a Percoll gradient (GE Healthcare Life Sciences, Piscataway, NJ) using a previously described method.[23] In